ENHANCEMENT OF PHENOLIC CONTENT AND ANTIOXIDANT PROPERTIES THROUGH CHEMICAL AND BIOCHEMICAL REATMENT OF LIGNIN-RICH RICE STRAW RESIDUE

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Valorization of lignin in biorefineries is important as this can lead to enhancement of chemical and physical properties of lignin, making it suitable for applications in polymers, composites and adhesives. Lignin valorization requires overcoming recalcitrance and tailoring for different uses. Here, lignin was extracted from rice straw lignin-rich residue at pH 12, using 4% magnesium oxide and 6% sodium hydroxide on a dry w/w basis. The extracted lignin-rich stream, which had a pH in the range of 9-10, was used further for biochemical transformation. Towards that end, *Pseudomonas putida*, *Sphingomonas paucimobilis*, *Pseudomonas fluorescens* and *Bacillus tequilensis* were screened for their capability of aromatic dye decolourization. The results of the study show an increase in the total phenolic content in lignin between 50% and 55% after 144 h of treatment with *Pseudomonas putida*. Concomitant with the increase of phenolics, a four-fold increase in antioxidant activity in gallic acid equivalents was also observed when *Pseudomonas putida* was used.

Keywords: rice straw, *Pseudomonas putida*, total phenolic content, lignin antioxidant

INTRODUCTION

After cellulose and hemicelluloses, lignin is the most abundant bio-based aromatic heteropolymer available on the earth. Because lignin contains a large number of aromatics, it holds enormous promise as a natural source for applications in bio-based polymers, biofuels and other aromatic value added applications, such as in phenol-based resins, bio-binder in bitumen, and natural polymer blends.

The total amount of lignin produced globally per year is approximately 50-70 million tons.[1] A large fraction (98%) of this lignin is generated from the paper and pulping industries, which follows 'lignin first' protocol of extracting lignin from the softwood and hardwood biomass. For the separation of lignin, the paper and pulps industry employ established methods, such as sulfite, soda and Kraft pulping.

Of the various methods for lignin extraction, the most employed is the Kraft process, which involves digestion of softwood chips with an aqueous solution of sodium sulfide and sodium hydroxide at an elevated temperature and pressure.[2] Kraft lignin has approximately 3% sulfur present, while lignosulfonate has around 8% to 12% sulfur.[3] During the Kraft pulping process, a huge amount of sulfur is generated and requires extensive water washing steps to clean the cellulose pulp for further applications. The sulfite process involves the use of sulfur dioxide (SO_2) and bisulfite salts to produce lignosulfonate, which is used extensively in the cement industry.[3] Soda pulping involves the use of sodium hydroxide with anthraquinone as a catalyst at elevated temperature and pressure.[3]

Most research on the valorization of lignin has focused much on the use of sulfur containing lignin. Only limited studies are available on the potential of sulfur-free lignin, which can be generated from the 2nd generation biorefineries aimed toward making cellulosic ethanol as the major products. With rapidly rising production to meet the biofuel requirements in the future and a projected annual production of 1.3 billion tons,

lignocellulosic biomass from 2G biorefineries provides a significant source of lignin.[3] The lignin that is usually left after the production of bioethanol has not been widely investigated and therefore, there is a great scope to valorize this lignin, which is the objective of the present work.

A challenge with regard to the utilization of lignin is the presence of β-o-4 linkages, methoxy groups and C-C linkages in lignin, which contributes to its recalcitrance and affects its reactivity for the subsequent processing into industry relevant derivatives. Conventional chemical methods for degradation of lignin involve the application of high temperature and pressures, toxic chemicals and other conditions, such as hydrothermal liquefaction, pyrolysis and gasification, which greatly increases complexity of the process. [4]

Microbial and fungal methods for lignin degradation have witnessed a great deal of progress in recent years, with great emphasis on white rot fungi, like *Phanerochaete chrysosporium*, *Trametes versicolor*, and bacteria such as *Streptomyces viridosporus*, *etc*.[8,9,10] These fungal and bacterial based systems are capable of lignin depolymerization with high efficacy at ambient temperature and an acidic to neutral pH range (between 5.5 and 6.5). The studies with bacteria are also interesting considering their rapid growth rate, along with the relative ease of genetic manipulation, which makes them potentially excellent candidates for microbial lignin depolymerization.

In most of the studies pertaining to bacterial degradation of lignin, experiments have been conducted at neutral pH with lignin coming from mostly paper and pulp, sulfonated lignin or lignin model compounds. There are not many studies conducted in the alkaline pH range, where there is the advantage of lignin being in solubilized form. This work investigates lignin valorization, wherein lignin can be extracted at alkaline pH, using chemicals, but the depolymerization can happen through the use of bacterial systems.

EXPERIMENTAL

Materials

Rice straw lignin-rich residue (RSSC) was obtained from the pilot plant of Praj Matrix, Pune, India. Magnesium oxide (≥99.0 w/w % purity), hydrogen peroxide solution (35 w/v % purity) and sodium hydroxide pellets (≥98.0 w/w % purity) were purchased from Fisher Scientific. Aromatic dyes Phenol red, Azure B and Malachite green, as well as potassium persulfate along with sulfuric acid (≥93 w/v % purity) were purchased from LobaChemi. Luria Bertani broth, yeast extract powder and pure peptone powder were purchased from HiMedia. Gallic acid and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (AR graded) were purchased from Sigma Aldrich.

Extraction and purification of lignin from rice straw lignin-rich residue

Lignin-rich rice straw residue was generated from rice straw using Praj's Enfinity® process.[11] The process first involves shredding of the rice straw to a particular size (10 mm), and then pretreatment is done at high temperature and pressure in a continuous pilot plant pretreatment reactor.[12] Following the pretreatment, rice straw was subjected to enzymatic hydrolysis for the conversion to hexose and pentose sugars, which were subsequently fermented to ethanol. The ethanol was distilled off and the solid liquid separation was conducted; this solid is rich in lignin, along with ash, some proteins and trace amounts of simple sugars. This solid lignin-rich residue was used as the feed raw material for extraction of lignin and its compositional analysis is provided in Table 1. The average size of lignin-rich rice straw residue is approximately 100 microns.

Table 1
Compositional analysis of rice straw lignin-rich residue after ethanol production in a 2nd generation biorefinery

Parameter	Value (%w/w)
Total solids	91.15
Ash	31.18
Proteins	10.09
Glucose	6.01
Xylose	0.80
Arabinose	0.10
Total lignin	42.03

Table 2
Compositional analysis of L2 extracted liquid lignin

Parameter	Value (%w/w)
Total solids	6.69
Ash	5.70
Proteins	18.22
Glucose	4.50
Xylose	0.60
Arabinose	Nil
Total lignin	68.48

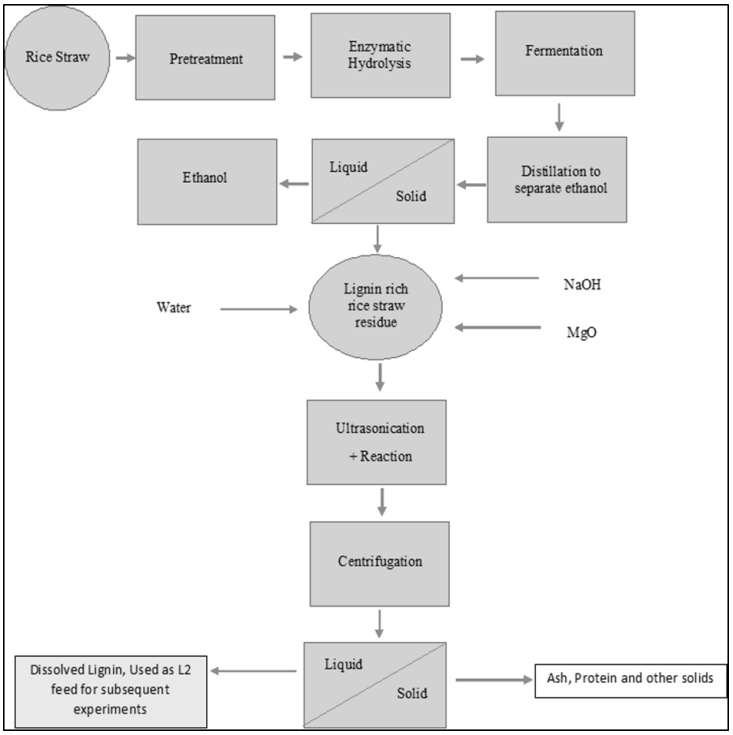

Figure 1: Process diagram for the extraction of lignin from residual biorefinery solid

Rice straw lignin-rich solid residue was added to reverse osmosis purified (RO) water on a 1:10 w/w basis. Reverse osmosis purified water was used to avoid possible interference of any impurities in the lignin extraction process, ionic or otherwise. Sodium hydroxide pellets were added gradually till the pH was stable at 12 before the addition of MgO. The slurry was placed into a 10 L stirred autoclave reactor (Amar, India) and was heated for up to ~45 minutes to reach a set point of 150 °C, after which it was maintained for an additional 1 hour with continuous mechanical agitation (100 RPM). At the end of the reaction, the slurry was recovered in a vessel and cooled to room temperature. The pH of the slurry was recorded with an Orion star A211 pH meter and the average value was found to be 9.5. The cooled reaction slurry was subjected to filter centrifugation at 5000 RPM for 30 minutes for solid-liquid separation. Filtration was done through a polypropylene felt bag with a pore size of 5 microns. The resulting slurry was filtered under identical conditions as the reaction mixture. The supernatant for the second filtration (labeled 'L2') contained most of the alkali-soluble lignin and was used as the feed for all the bacterial degradation studies. Figure 1 summarizes the procedure of obtaining L2 lignin from agricultural rice straw.

Use of aromatic dyes as indicators for screening bacterial strains

Phenol Red, Azure B and Malachite Green dyes were added to sterile nutrient broth at a final concentration of 100 mg/L in 500 mL conical flasks each for the screening of the activity of *Pseudomonas putida*, *Sphingomonas paucimobilis*, *Pseudomonas fluorescens* and *Bacillus tequilensis*. For each set of flasks for a single dye, a control flask, which was not inoculated with any bacteria, was maintained to confirm the dye decolorizing capacity of the bacterial strains to be screened. Following inoculation, the flasks were incubated for 24 h, after which each

sample was subjected to UV-Vis spectrophotometry. The wavelengths used in spectrophotometry (λ_{max}) were 435 nm for Phenol Red, 650 nm for Azure B and 615 nm for Malachite Green, in accordance with their maximum absorbance wavelength, along the UV-Vis spectrum.[13] The absorption of the flasks for each dye were recorded and compared before (0 h) and after (24 h) inoculation.

Screening for survivability and growth rate of bacterial strains

Pseudomonas putida, *Sphingomonas paucimobilis*, *Pseudomonas fluorescens* and *Bacillus tequilensis* were further screened for their ability to perform under alkaline conditions of L2 lignin media. The lignin media was prepared by dissolving L2 in Milli-Q water in a ratio of 1:10. This was dispensed into 4 flasks of 500 mL volume, one intended for each bacterial strain. To this, following the autoclave treatment, glucose solution was added, so the final concentration of glucose was 2% w/v in the total volume.

Seed inoculum for each bacterial strain was prepared by inoculating flasks with Luria-Bertani broth and incubating at 30 °C for 24 h. The seed inoculum was kept at 10% volume when transferring over to the L2 flasks. The L2 flasks were incubated under identical conditions of 30 °C. The samples were recovered over intervals of 24 h over a total period of 144 h. The samples were plated on Luria-Bertani agar plates and after incubation under identical conditions for 24 h; the colonies thus-formed were counted and documented for comparison.

Degradation of alkaline lignin using *Pseudomonas putida*

L2 lignin from the lignin extraction step was utilized as the feed for *Pseudomonas putida* bacteria. L2 was diluted in Milli-Q water so that the final concentration of the media after addition of all components would be 10% L2 on a v/v basis. 40% (w/v) NaOH solution was added to increase the pH of the solution to 9.5. This lignin solution was autoclaved at 121 °C for 20 minutes. Three different batches were considered:

1) Non-enriched (with only L2 lignin as a sole carbon source);
2) L2 lignin media enriched with yeast extract at 2 g/L;
3) L2 lignin media enriched with yeast extract at 2 g/L and peptone at 1 g/L.

This feed was charged into a New Brunswick Bioflo/CelliGen 115 1L batch fermenter maintained at 30 °C with 150 RPM.

The seed culture of pure *Pseudomonas putida* was cultivated in Luria Bertani broth for 24 h. The seed was added as 10% of the total media volume through a port. 20 mL of the media was siphoned out through the sampling port in 24 h interval up to a time point of 144 h. Batches of yeast extract only and yeast extract with peptone were compared to a control group with only 10% lignin solution.

Estimation of protein of lignin samples

In accordance with NREL protocols, 0.5 g of the samples were digested in 72% sulfuric acid, along with 8 g potassium sulfate and 0.5 g copper sulfate at 410 °C for 1 h.[16] This protein digest was subjected to a steam distillation (Kjeltec 8200) and the distillate was transferred into a phenolphthalein indicator solution, leading to a change in color to teal blue. This solution was back titered using 0.1N HCl to neutralize the base. The amount of HCl utilized, in mL, was used in calculation of the protein content. The protein content was calculated as:

$$P(w/w\%) = \frac{[(X - X')(14.007)(6.25)(100)N]}{W} \quad (1)$$

where P is the percentage of protein content of the sample (w/w % basis), X is the titer for the sample in mL, X' is the titer for the blank in mL, N is the normality of HCl used to back-titer the solution, W is the weight of the sample used in mg, 14.007 u is the atomic mass of nitrogen and 6.25 is the nitrogen-to-protein conversion factor.

Estimation of carbohydrates and lignin

Carbohydrates, lignin, ash, and protein in the lignin-rich residue obtained from the 2G ethanol process was measured using standard NREL methods. In accordance with NREL protocols, the lignin-rich residue samples were washed with Milli-Q water through a vacuum filter to remove any trace extractives and non-structural carbohydrates.[17] The fixed amount of each sample was transferred to their respectively labeled borosilicate glass bottles. To this, a fixed amount of 72% of sulfuric acid was added to each bottle and the samples were incubated in a water bath at 30 °C for 1 h. Following the warm water bath, the acid within the bottles was diluted with Milli-Q water. For reference, Sugar Recovery Standards (SRS) for glucose, xylose and arabinose were placed in their own bottles along with identical concentration of sulfuric acid for hydrolysis. All glass bottles were placed in a safety rack within an autoclave. The autoclave temperature was set to 121 °C for 1 h.

After autoclaving, an aliquot of the liquid was taken for determination of carbohydrates and acid soluble lignin. The liquor aliquot was analyzed using a UV-Vis spectrophotometer at 240 nm wavelength in quartz cuvettes with deionized water as standard for quantification of acid soluble lignin. For quantification of structural carbohydrates, a Calibration Verification Standard (CVS) was prepared for each individual carbohydrate type (glucose, arabinose and xylose). Following neutralization with calcium carbonate, the liquor aliquot was passed through an HPLC for quantification of glucose, xylose and arabinose.

For measuring insoluble lignin, the solid residue following vacuum filtration of the hydrolyzed liquor was washed with deionized water and dried in a hot air

oven. The dried solids were weighed in an analytical balance (Mettler-Toledo ME104, of 0.01 mg readability) for a base-line measure and subjected to incineration in a muffle furnace at 575 °C for 6 h. The differences between the weights of dried lignin and ash were considered, along with protein content, for calculating insoluble lignin.[17]

Quantification of total phenolic content (TPC)

The total phenolic content of the lignin samples was determined using the Folin-Ciocalteu reagent method, as performed by Rumf *et al.* with minor changes.[18] L2 Lignin samples of all 3 batches from every time point at 24 h intervals were further diluted with Milli-Q water in a ratio of 1:2. 60 µL of the diluted samples were made up with Milli-Q water to 5 mL and treated with 300 µL Folin–Ciocalteu reagent. Following an incubation of 8 minutes, 900 µL of sodium carbonate was added, followed by mild stirring and incubation at 20 °C for 90 minutes. The samples were subjected to UV-Vis spectrophotometry on a Lab India UV3200 double beam spectrophotometer at 765 nm wavelength with Milli-Q water as blank. All samples were taken in triplicates.

Gallic acid was used as a standard with known gradually increasing concentration from 0 mg/mL (blank) to 1000 mg/mL. A standard curve was obtained for gallic acid in $Y = mX + b$ format, where Y is the absorbance unit at 765 nm wavelength and X is the concentration of gallic acid in mg/ mL.

Absorption values of subsequent lignin samples were substituted in the obtained standard curve equation to obtain the concentration in gallic acid equivalent mg/mL or GAE mg/mL.

A separate comparison study was made by incubating a sterile 10% L2 lignin batch for 144 h under identical temperature, agitation and pH conditions to the previous conventional batches. Every 24 h, the lignin was sampled and again analyzed for TPC to quantify the effect of *Pseudomonas putida* on the amount of phenolic content as a function of time.

ABTS•+ radical scavenging antioxidant assay

The antioxidant activity of lignin samples at set time points was determined using the ABTS assay with reference to Oihana *et al*.[19] with some modifications. L2 lignin at various times points: 0 h, 48 h and 144 h from all 3 types of batches were subjected to precipitation by addition of 93% H_2SO_4 until the pH of 2.5 was obtained. The slurries obtained from this precipitation step were centrifuged using an Eppendorf Centrifuge 5424 R at 16000 RPM for 5 minutes to separate solid lignin from liquid the supernatant. Solid lignin was dried in a hot air oven at 70 °C overnight. Dried solid lignin from the time points of each type of batch was separately dissolved in water of pH 9.0 (using NaOH) of known concentrations of 1.25 mg/mL, 2.5 mg/mL and 5 mg/mL.

For the ABTS•+ radical working solution, a 7 mM ethanolic solution of ABTS was prepared. To this, an aqueous solution of potassium sulfate ($K_2S_2O_8$) was added, so that the final concentration of $K_2S_2O_8$ was 2.45 mM. This stock solution was kept in darkness at room temperature for 16 h. The working solution was prepared by diluting part of the stock solution with ethanol till an absorbance of 0.7000 ± 0.02 was obtained at 734 nm with ethanol as blank.

Triplicates of 1.5 mL sterile Eppendorf tubes for selected time points of control, yeast extract only and yeast extract with peptone containing 1 mL of the working solution were placed on a 96 well-plate. A Finnpipette F2 Multichannel pipette was used to dispense 10 µL of all 3 concentrations of lignin along with a blank (ethanol) for each time point. The mixtures were incubated in darkness for 6 minutes. After incubation, the samples were immediately checked for their absorbance at 734 nm in a Mettler-Toledo Easy UV spectrophotometer. The reduction in absorbance compared to the blank was used to obtain the degree of decolourization using the following formula:

$$D(\%) = \frac{(A' - A)}{A'} \quad (2)$$

where D is the decolourization percentage, A' is the absorbance for the blank at 734 nm wavelength and A is the absorbance for the sample at 734 nm wavelength.

Statistical analysis for total phenolic content and antioxidant activity

The standard error for the total phenolic content of all batches was taken in triplicate. The following are the samples for which the standard error is mentioned in this work: (a) sterile lignin, (b) lignin without yeast extract or peptone, (c) lignin with yeast extract and (d) lignin with yeast extract and peptone. The average values of triplicates for each time point were used for the plotting of the graphs. Using the following formula, the standard error for each time point was calculated:

$$SE = \left(\frac{\sigma}{\sqrt{n}}\right) \quad (3)$$

where σ is the standard deviation of the total phenolic content value from the average and n is the number of repeats (in this case, n = 3). Table 3 shows the standard error for each time point for all batches.

For antioxidant activity, a plot of increase in decolourization as a function of concentration was used to obtain the IC_{50} values of each time point. IC_{50} is the concentration of a sample required to cause a reduction of absorbance of 50% of the ABTS•+ solution at the same wavelength.

Gallic acid was used as a reference for the antioxidant assay. A standard curve for gallic acid involved an identical setup to the lignin samples in a 96 well-plate with known concentrations of 0 (blank ethanol), 12.5 µM, 25 µM, 50 µM, 100 µM, 200 µM, 400 µM and 800 µM. The equation of the standard

curve was obtained in $Y = mX + b$ format and used to obtain the gallic acid equivalent antioxidant activity of the lignin samples (GAEAC). The GAEAC was obtained using the formula:

$$GAEAC\ \left(\frac{mg}{mg}\right) = \frac{\left[\frac{(D-b)}{m}\right]}{C} \quad (4)$$

where D is the decolourization percentage of the sample, b is the intercept and m is the slope for the standard curve equation for gallic acid and C is the concentration of the sample in mg/mL. Similar to the total phenolic content values, the standard error for the antioxidant activity in gallic acid equivalents was calculated and shown in Table 4.

The IC_{50} for each sample time point was calculated by plotting a graph of decolourization % as a function of concentration. After the $Y = mX + b$ formula for each individual time point was obtained, the concentration required (X mg/mL) for which 50% decolourization of ABTS•+ radical solution was achieved (Y = 50) was calculated. Thus, a lower IC_{50} and higher GAEAC value could be credited to a higher antioxidant activity and, in turn, higher phenolic content, as supported by Duanab *et al.*,[20] and Jevgenija *et al.*[21] Table 4 shows the antioxidant activity of samples in gallic acid equivalent µg/mg of sample, along with their corresponding IC_{50} values.

Table 3
Standard error values for total phenolic content across 144 h for sterile lignin, non-enriched lignin, lignin enriched with yeast extract only and lignin enriched with yeast extract and peptone

	Standard error (PPM)			
hour	Sterile	Non-enriched	Yeast extract only	Yeast extract with peptone
0	± 13.1	± 26.5	± 32.3	± 2.9
24	± 11.3	± 2.2	± 35.5	± 17.8
48	± 4.9	± 13.6	± 20.1	± 17.6
72	± 2.6	± 30.9	± 36.9	± 16.1
96	± 10.1	± 2.0	± 22.6	± 34.7
120	± 3.7	± 16.4	± 9.9	± 18.0
144	± 2.0	± 15.0	± 38.8	± 8.5

Table 4
Gallic acid equivalent antioxidant activity and the IC_{50} values for all groups at 0 h and 144 h time points

Sample	GAEAC (µg gallic acid/mg)	IC50 (mg/mL)
Control 0 h	3.150 ± 0.086	12.465 ± 0.490
Control 144 h	1.770 ± 0.040	11.238 ± 0.140
Yeast extract only 0 h	1.720 ± 0.033	12.296 ± 0.280
Yeast extract only 144 h	5.460 ± 0.095	5.253 ± 0.360
Yeast extract with peptone 0 h	1.120 ± 0.044	19.494 ± 0.240
Yeast extract with peptone 144 h	4.950 ± 0.110	6.634 ± 0.520

RESULTS AND DISCUSSION

Extraction of lignin from rice straw lignin-rich residue

After ethanol production, rice straw, also called rice straw spent cake, is mostly used as fuel for boilers. As such, it has very little market value. Its use as feedstock for lignin extraction adds potential for valorization of an otherwise unremarkable byproduct. Unlike the lignin obtained from the paper and pulp industries, which is extracted on a 'lignin first' basis, biorefinery lignin-rich residue is only obtained post-ethanol production. Hence, the lignin-rich residue in biorefineries has a significant amount of ash and other fermentation related impurities, which may inhibit certain microbial processes from utilizing lignin to their fullest extent. Hence, a chemical process for extracting lignin from the solid residue was utilized to add value to the lignocellulosic biomass and improve carbon utilization. MgO was added to improve the conversion percentage in depolymerization of lignin and reduce the presence of ash in the L2 stream.[23] The addition of MgO also reduced the presence of residual carbohydrates, such as glucose and xylose, in the liquid lignin (L2) stream. Bacterial degradation of lignin requires liquid media for growth and phenolic enhancement. Hence, the L2 stream was utilized in its liquid state, instead of drying into a powder.

As shown in Tables 1 and 2, the extracted lignin L2 stream showed about ~63% increase in the total lignin content, as compared to the lignin-rich residue feed, while also significantly reducing the amount of ash (~81%).

Microbial screening for aromatic dye decoloration and selection of microbial strain

Pseudomonas putida, *Pseudomonas fluorescens*, *Sphingomonas paucimobilis* and *Bacillus tequilensis* were screened for lignin degradation.[24,25,26]

Azo dyes were selected on the basis of their structural similarity to polymeric lignin, as loss of dye color post-inoculation would indicate lignolytic activity.[25]As shown in Figures 2, 3 and 4, colour removal was achieved by all bacterial strains to varying degrees. The highest degree of decolourization for Malachite Green was shown by *Pseudomonas putida* at 88.4% after 24 h. *Pseudomonas putida* showed decolourization activity to a lesser degree against Phenol Red and Azure B at 25.1% and 24.2%, respectively. Phenol Red showcased the highest resistance to degradation, while Malachite Green had the most significant degradation by a very large margin. Rapid decolourization of azo-dye Malachite Green correlates to laccase activity of *Pseudomonas fluorescens* and *Pseudomonas* putida.[26,27]

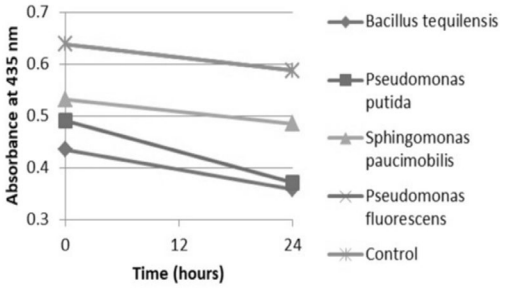

Figure 2: Dye decoloration of Phenol Red over 24 hours when compared to a sterile control

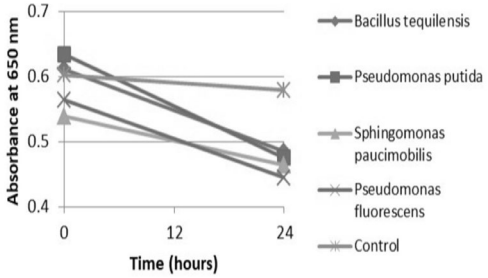

Figure 3: Dye decoloration of Azure B over 24 hours when compared to a sterile control

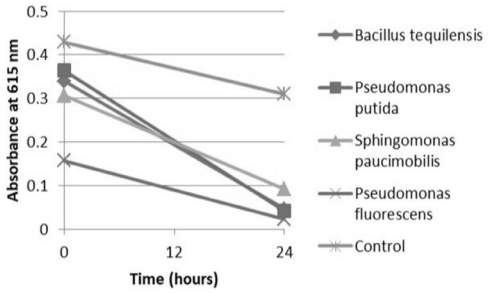

Figure 4: Dye decoloration of Malachite Green over 24 hours when compared to a sterile control

Lignin-derived phenolic compounds have also been reported to inhibit enzymatic activity and growth.[19] Hence, the selection process requires a robust, phenolic resistant strain capable of growing in harsh, alkaline and salt rich conditions. Due to the combined factors of both *Pseudomonas putida* and *Pseudomonas fluorescens* robustness, adaptability in alkaline conditions, fast growth rate and resilience to highly phenolic environment, along with lignolytic activity, they were chosen as the strains for increasing the phenolic content of biorefinery lignin.

Screening for survivability and growth rate of bacterial strains

As shown in Figure 5, *Pseudomonas putida* and *Pseudomonas fluorescens* showed the highest growth in L2 lignin media, with a nearly identical growth pattern over 144 h. *Bacillus tequilensis* showed growth at a lower degree up to 48 h, after which the growth drastically decreased. However, *Sphingomonas paucimobilis* showed very low growth throughout the 144 h period. The maximum of the growth curve for each strain was achieved at around 48 h, after which the colony count plateaued. Owing to the results thus

obtained, *Pseudomonas putida* was chosen for further experiments.

Growth of *Pseudomonas putida* in both neutral and alkaline conditions

Pseudomonas putida showed great robustness regarding growing in both alkaline and neutral conditions in a 500 mL flask over 144 h with 150 RPM agitation. The growth curve provided in Figure 6 showed very similar trends with similar growth amount.

Pseudomonas putida, showing ability to metabolize aromatic carbon sources, catabolizes monomeric phenols from lignin, incorporating them into the Krebs cycle, leading to the conversion into pyruvic acid and oxaloacetate in accordance with Notonier *et al*.[30]

Nitrogen sources like yeast extract and peptone increase bacterial enzyme synthesis, as affirmed by Huda *et al.* and, in turn, the increase in synthesis of bacterial o-demethylases increases the phenolic content of the culture, as compared to the control group.[31] Like the results for growth rate of bacteria, the yeast extract alone shows greater increase in the overall total phenolic content than when the culture was enriched with both yeast extract and peptone.

Effect of yeast extract and peptone on growth of *Pseudomonas putida*

Figure 7 shows the growth curve for *Pseudomonas putida* under the control conditions, with yeast extract only and with yeast extract and peptone. When the L2 medium was enriched with yeast extract only, as well as when yeast extract with peptone were added to the medium, the bacterial growth rate was noticeably increased. The control flask showed not only lower cell growth, but also a later onset of logarithmic growth phase.

Total phenolic content

Pseudomonas putida showed activity through increasing the total phenolic content of 10% lignin over 144 h. The total phenolic content of the experimental groups were labeled as sterile lignin, non-enriched L2, L2 enriched with yeast extract and L2 enriched with yeast extract and peptone over 144 h; the values for which are provided in Figure 8. The total phenolic content of the control batch showed a minor increase for the first 48 hours, reaching a maximum of a 9.77% increase in TPC. However, the phenolics gradually decreased over time until a final value of 3.92% increase from the initial value.

The lignin media enriched with yeast extract only (2 g/L) showed an overall increase in TPC values throughout the duration until a final value of 50.67% increase in the TPC was achieved. While it was expected for the yeast extract (2 g/L) with peptone (1 g/L) to lead to an increase in the TPC even further, the final value of increase in TPC was 15.8%. The blank sterile lignin showed nearly constant TPC values throughout the 144 h time period with minute changes.

Both yeast extract only and yeast extract with peptone batches showed an identical pattern of increase in TPC, with a distinct decrease at 24 h and 120 h time points, while the control batch had a much more sporadic pattern.

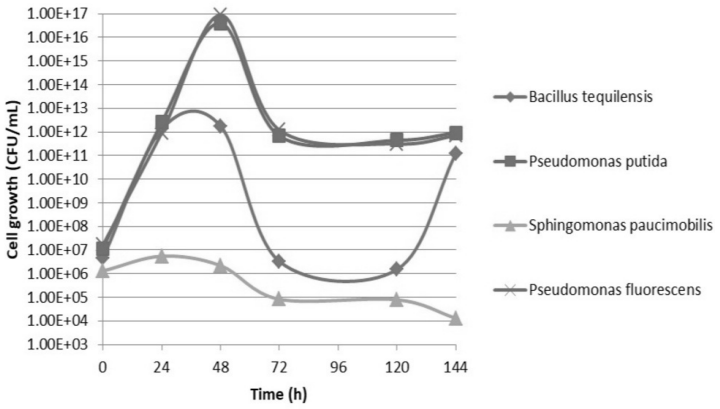

Figure 5: Bacterial strains *Pseudomonas putida*, *Pseudomonas fluorescens*, *Sphingomonas paucimobilis* and *Bacillus tequilensis*, grown on 10% lignin media with 2% glucose over 144 hours at pH 9.0 (lines in the graph are purely for guiding along their individual timepoints and have not been fitted)

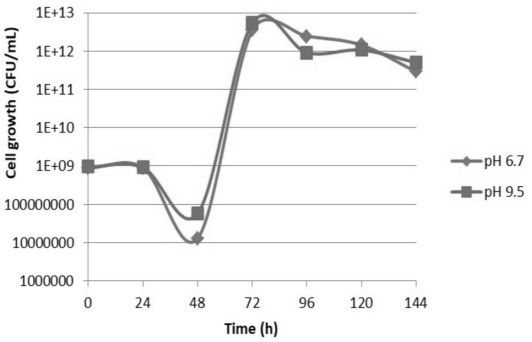

Figure 6: Growth curves for *Pseudomonas putida* over 144 hours at starting pH of 6.7 and 9.5

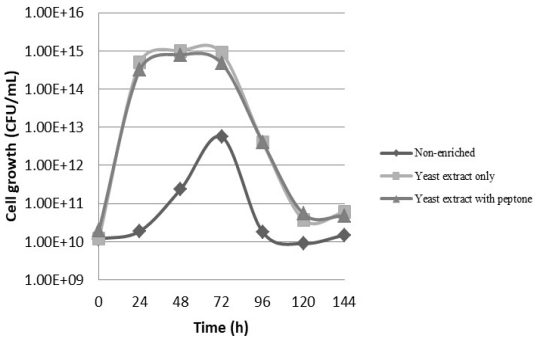

Figure 7: CFU/mL of *Pseudomonas putida* over 144 hours for addition of yeast extract only and yeast extract with peptone in a logarithmic scale

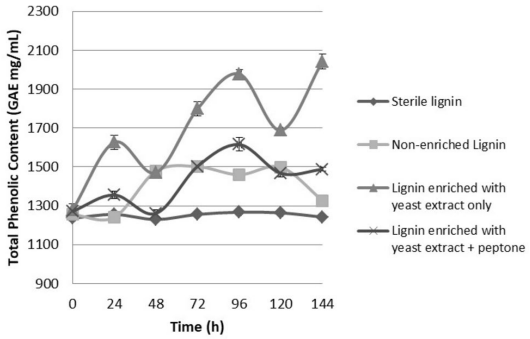

Figure 8: Total phenolic content measured in gallic acid equivalent mg/mL of *Pseudomonas putida* fermentation batches for control, and with the addition of yeast extract only and yeast extract with peptone over 144 hours

ABTS•+ radical scavenging antioxidant assay and correlation with total phenolic content

To understand the increase in the antioxidant capacity of the lignin samples after 144 h under control conditions, with yeast extract addition only and with yeast extract and peptone addition, the ABTS•+ radical scavenging activity was investigated, and known antioxidant gallic acid was used as a standard. The concentration of sample required to achieve 50% decolourization of ABTS•+ was calculated and plotted.

Figure 9 shows the change in IC_{50} values of samples for all three streams at the starting point (0 h) and at the end point (144 h). While antioxidant activity remained nearly unaffected for the control stream, the yeast extract, as well as the yeast extract plus peptone enriched streams showed a significant decrease in their IC_{50}.

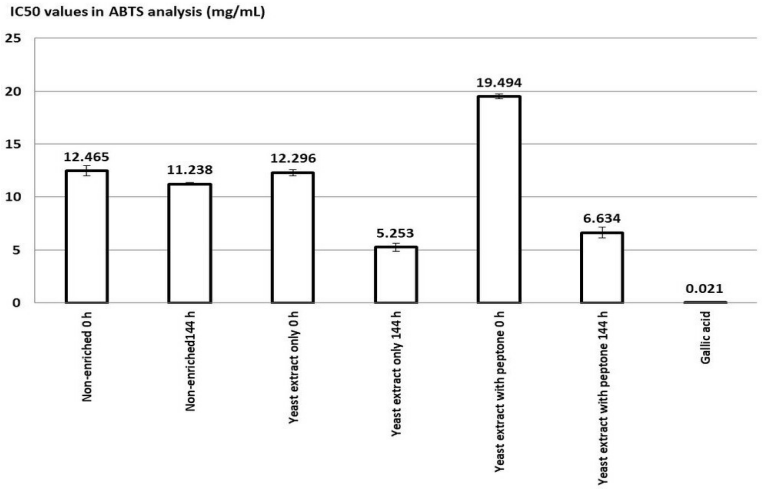

Figure 9: IC_{50} for all samples as compared to gallic acid standard

CONCLUSION

Lignin was extracted from 2G ethanol plant lignin-rich rice straw residue, with minimal presence of ash and carbohydrates in the extract stream. *Pseudomonas putida* displayed significant lignolytic activity through decoloration of lignin-mimicking dyes, Phenol Red, Azure B and Malachite Green to varying degrees. *Pseudomonas putida* showed capability of growth in alkaline pH on par with neutral pH under identical temperature conditions. Nitrogen sources, such as the yeast extract, were shown to greatly increase both growth rate and lignin degradation in *Pseudomonas putida*. Total phenolic content increased significantly when the media were enriched with yeast extract. The addition of peptone was expected to improve the lignin degradation ability of *Pseudomonas putida*, but it was not observed to. The increase in total phenolic content was supported with an increase in antioxidant activity in lignin samples. Bacterial degradation of alkaline lignin enriched with organic nitrogen sources shows promise for subsequent applications.

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